REPUBLIC OF SOUTH AFRICA
PATENTS ACT, 1978
APPLICATION FOR A PATENT AND
ACKNOWLEDGEMENT OF RECEIPT
(Section 30(1) Regulation 22)

FORM P.1
(to be lodged in duplicate)

REPUBLIC OF SOUTH AFRICA
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THE GRANT OF A PATENT IS HEREBY REQUESTED BY THE UNDERMENTIONED APPLICANT ON THE BASIS OF THE PRESENT APPLICATION FILED IN DUPLICATE.

A & A REF: 117131

| PATENT APPLICATION NO. | |
|---|---|
| 21 01 | 888127 |
| 71 | FULL NAMES(S) OF APPLICANT(S) |

AMERICAN CYANAMID COMPANY

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54 TITLE OF INVENTION

TARGETED FORMS OF METHYLTRITHIO ANTITUMOR AGENTS

Only the items marked with an "X" in the blocks below are applicable.

- [X] The Applicant claims priority as set out on the accompanying Form P.2
- [ ] The application is for a patent of addition to patent application no. |21|01|
- [ ] This application is a fresh application in terms of section 37 and based on Application No. |21|01|

THIS APPLICATION IS ACCOMPANIED BY:

- [X] ~~A single copy of a provisional or~~ two copies of a complete specification of ..51..pages.
- [X] Drawing of ..3... sheets. (INFORMAL) (GRAPHS)
- [X] Publication particulars and abstract (Form P.8 in duplicate) (for complete only).
- [ ] A copy of Figure......of the drawings (if any) for the abstract (for complete only).
- [ ] An assignment of invention.
- [ ] Certified priority document(s) (State quantity):.........
- [ ] Translation of the priority document(s).
- [ ] An assignment of priority rights.
- [ ] A copy of Form P.2 and the specification of RSA Patent Application No. |21|01|
- [X] A Form P.2 in duplicate.
- [X] A declaration and power of attorney on Form P.3.
- [ ] Request for ante-dating on Form P.4.
- [ ] Request for classification on Form P.9.
- [ ] Request for delay of acceptance on Form P.4.
- [ ]

74 ADDRESS FOR SERVICE: Adams & Adams, Pretoria

DATED THIS 28TH DAY OF OCTOBER 19 88

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APPLICANTS PATENT ATTORNEYS

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A. & A. Ref. No. 117131

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FORM P7

REPUBLIC OF SOUTH AFRICA
Patents Act, 1978

# COMPLETE SPECIFICATION

(Section 30 (1) - Regulation 28)

| OFFICIAL APPLICATION NO. | |
|---|---|
| 21 01 | 888127 |

| LODGING DATE | |
|---|---|
| 22 | 28 OCTOBER 1988 |

| INTERNATIONAL CLASSIFICATION | |
|---|---|
| 51 | A61K C07C C07D C07G |

| FULL NAMES(S) OF APPLICANT(S) | |
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| TITLE OF INVENTION | |
|---|---|
| 54 | TARGETED FORMS OF METHYLTRITHIO ANTITUMOR AGENTS |

30,682-02

Title: TARGETED FORMS OF METHYLTRITHIO ANTITUMOR AGENTS

~~This application is a continuation-in-part of co-pending application Serial No. 07/114,940, filed October 30~~, ~~1987~~.

## SUMMARY OF THE INVENTION

The invention describes carrier-drug conjugates of the disulfide analogs of the $\alpha_1$, $\alpha_2$, $\alpha_3$, $\alpha_4$, $\beta_1$, $\beta_2$, $\gamma_1$ and $\delta$ components of the LL-E33288 complex as well as the disulfide analogs of BBM-1675, FR-900405, FR-900406, PD 114759, PD 115028, CL-1577A, CL-1577B, CL-1577D, CL-1577E and CL-1724 antitumor antibiotics. The carrier portion of the conjugate is a mono- or polyclonal antibody, their fragments, chemically or genetically manipulated counterparts, growth factors or steroids. The invention includes the method of using the carrier-drug conjugates as well as their process of manufacture.

## DESCRIPTION OF THE DRAWINGS

Figure I: The ultraviolet spectrum of the antitumor antibiotic designated as LL-E33288$\gamma_1^{I}$.

Figure II: The proton magnetic resonance spectrum of the antitumor antibiotic designated as LL-E33288$\gamma_1^{I}$.

Figure III: The infrared spectrum of the antitumor antibiotic designated as LL-E33288$\gamma_1^{I}$.

## DETAILED DESCRIPTION OF THE INVENTION

The family of antibacterial and antitumor agents, known collectively as the LL-E33288 complex are described and claimed in copending U.S. patent application, Serial No. 009,321, filed January 30,1987 and are used to prepare

$$\begin{array}{l}\mathrm{Tu}\text{-}(\mathrm{Z}\text{-}\mathrm{Sp}\text{-}\mathrm{SS}\text{-}\mathrm{W})_m \\ | \\ (\mathrm{Y})_{n-m}\end{array}$$

wherein Tu, Y, Sp, W, and n are as hereinbefore defined, and Z is formed from covalent reaction of the groups Q and Y directly or after subsequent reduction, and Z is -CONH-, -CONHN=CH-, $-CONHNHCH_2-$, or

$$\begin{array}{l} \qquad\qquad\quad | \\ -\mathrm{NHCOCH_2}\text{-}\mathrm{CH} \\ \qquad\qquad\quad | \\ \qquad\qquad\quad (\mathrm{CH_2})_{0,1} \\ \qquad\qquad\quad | \\ \qquad\qquad\quad \mathrm{CO_2H}\end{array}$$

and m is 0.1 to 15, and said method comprising administering to a mammal, an effective oncolytic amount of said substance or composition.

11. A new process for preparing the targeted derivatives of

$$\begin{array}{l}\mathrm{Tu}\text{-}(\mathrm{Z}\text{-}\mathrm{Sp}\text{-}\mathrm{SS}\text{-}\mathrm{W})_m \\ (\mathrm{Y})_{n-m}\end{array}$$

of compounds of formula $CH_3SSS$-W, substantially as herein described and illustrated.

12. A new method of inhibiting growth of tumors in a mammal, substantially as herein described and illustrated.

13. A substance or composition for a new use in a method of treatment, substantially as herein described and illustrated.

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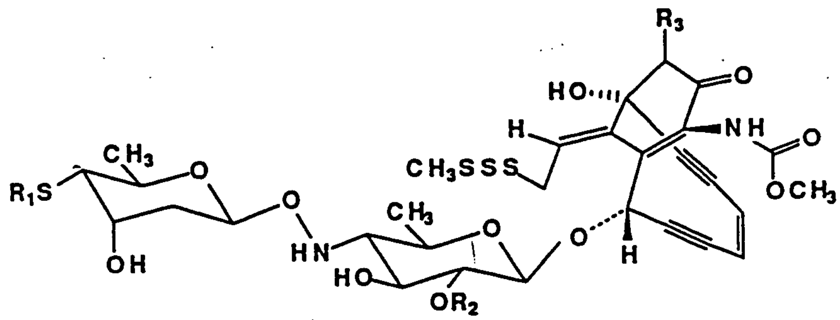
the disulfur antitumor agents which are starting materials for targeted forms of the antitumor agents of our invention.

The Serial No. 009,321 application describes the LL-E33288 complex, the components thereof, namely, LL-E33288$\alpha_1^{Br}$, LL-E33288$\alpha_1^{I}$, LL-E33288$\alpha_2^{Br}$, LL-E33288$\alpha_2^{I}$, LL-E33288$\alpha_3^{Br}$, LL-E33288$\alpha_3^{I}$, LL-E33288$\alpha_4^{Br}$, LL-E33288$\beta_1^{Br}$, LL-E33288$\beta_1^{I}$, LL-E33288$\beta_2^{Br}$, LL-E33288$\beta_2^{I}$, LL-E33288$\gamma_1^{Br}$, LL-E33288$\gamma_1^{I}$ and LL-E33288$\delta_1^{I}$, and methods for their production by aerobic fermentation utilizing a new strain of <u>Micromonospora</u> <u>echinospora</u> ssp <u>calichensis</u> or natural or derived mutants thereof. Serial No. 009,321 also discloses proposed structures for some of the above named components. These proposed structures are reproduced in Table 1.

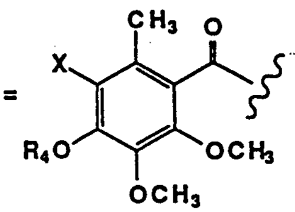
## Table 2

Proposed Structures for $CH_3$-SSS-W

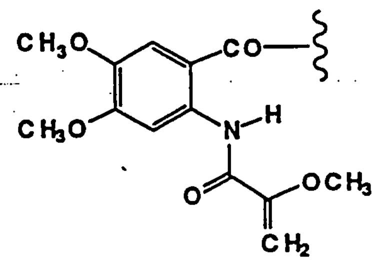
(wherein W is the substituent attached to $CH_3$-SSS- below)



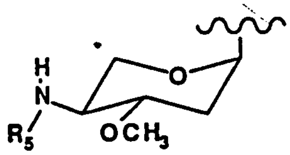

Ar$_1$ =



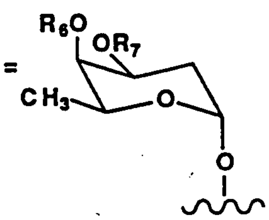

Ar$_2$ =



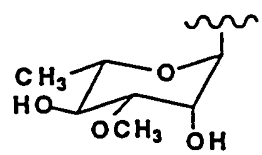

R$_{2'}$ =




R$_{3'}$ =




R$_{4'}$ =




| Designation | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ | $R_6$ | $R_7$ | X |
|---|---|---|---|---|---|---|---|---|
| LL-E33288$\alpha_2^{I}$ | $Ar_1$ | $R_{2'}$ | H | H | $C_2H_5$ | | | I |
| LL-E33288$\alpha_3^{I}$ | $Ar_1$ | H | H | $R_{4'}$ | | | | I |
| LL-E33288$\beta_1^{I}$ | $Ar_1$ | $R_{2'}$ | H | $R_{4'}$ | $(CH_3)_2CH$ | | | I |
| LL-E33288$\gamma_1^{I}$ | $Ar_1$ | $R_{2'}$ | H | $R_{4'}$ | $C_2H_5$ | | | I |
| LL-E33288$\delta_1^{I}$ | $Ar_1$ | $R_{2'}$ | H | $R_{4'}$ | $CH_3$ | | | I |
| LL-E33288$\beta_1^{Br}$ | $Ar_1$ | $R_{2'}$ | H | $R_{4'}$ | $(CH_3)_2CH$ | | | Br |
| LL-E33288$\gamma_1^{Br}$ | $Ar_1$ | $R_{2'}$ | H | $R_{4'}$ | $C_2H_5$ | | | Br |
| LL-E33288$\alpha_2^{Br}$ | $Ar_1$ | $R_{2'}$ | H | H | $C_2H_5$ | | | Br |
| LL-E33288$\alpha_3^{Br}$ | $Ar_1$ | H | H | $R_{4'}$ | | | | Br |
| Esperamicin $A_1$ | $CH_3$ | $R_{2'}$ | $R_{3'}$ | | $(CH_3)_2CH$ | H | $Ar_2$ | |
| Esperamicin $A_2$ | $CH_3$ | $R_{2'}$ | $R_{3'}$ | | $(CH_3)_2CH$ | $Ar_2$ | H | |
| Esperamicin $A_{1b}$ | $CH_3$ | $R_{2'}$ | $R_{3'}$ | | $CH_3CH_2$ | H | $Ar_2$ | |

Certain other antibiotics are useful in our invention, namely:

1) Esperamicin BBM-1675, a novel class of potent antitumor antibiotics. I. Physico-chemical data and partial structure. M. Konishi, _et_. _al_., J. Antibiotics, _38_, 1605 (1985). A new antitumor antibiotic complex, M. Konishi, _et_. _al_., U.K. Patent Application GB 2,141,425A, May 15, 1984.

2) New antitumor antibiotics, FR-900405 and FR-900406.I. Taxonomy of the producing strain. M. Iwami, _et_. _al_., J. Antibiotics _38_, 835 (1985). New antitumor antibiotics FR-900405 and FR-900406.II. Production, isolation, characterization and antitumor activity. S. Kiyoto, _et_. _al_., J. Antibiotics, _38_, 340 (1985).

3) PD 114759 and PD 115028, novel antitumor antibiotics with phenomenal potency. I. Isolation and characterization. R.H. Bunge, _et_. _al_., J. Antibiotics, _37_, 1566 (1984). Biological and biochemical activities of the novel antitumor antibiotic PD 114759 and related derivatives. D.W. Fry _et_. _al_., Investigational New Drugs, _4_, 3 (1986).

4) New antibiotic complex CL-1577A, CL-1577B produced by _Streptomyces_ sp. ATCC 39363. European Patent Application 0,132,082, A2.

5) CL-1577D and CL-1577E Antibiotic antitumor compounds, their production and use. U.S. Patent 4,539,203.

6) CL-1724 Antibiotic compounds, their production and use. U.S. Patent 4,554,162.

All of the information regarding BBM-1675, FR-900405, FR-900406, PD 114759, PD 115028, CL-1577A, CL-1577B, CL-1577D, CL-1577E and CL-1724 contained in the above citations is incorporated herein by reference. The complete structures of esperamicins $A_1$, $A_2$, and $A_{1b}$ (the BBM-1675 complex) have been reported, and these are included in Table 1. The physical characteristics of the above-named antitumor antibiotics indicate that they all are identical or very similar in structure to the esperamicins, and all contain a methyltrithio functional group.

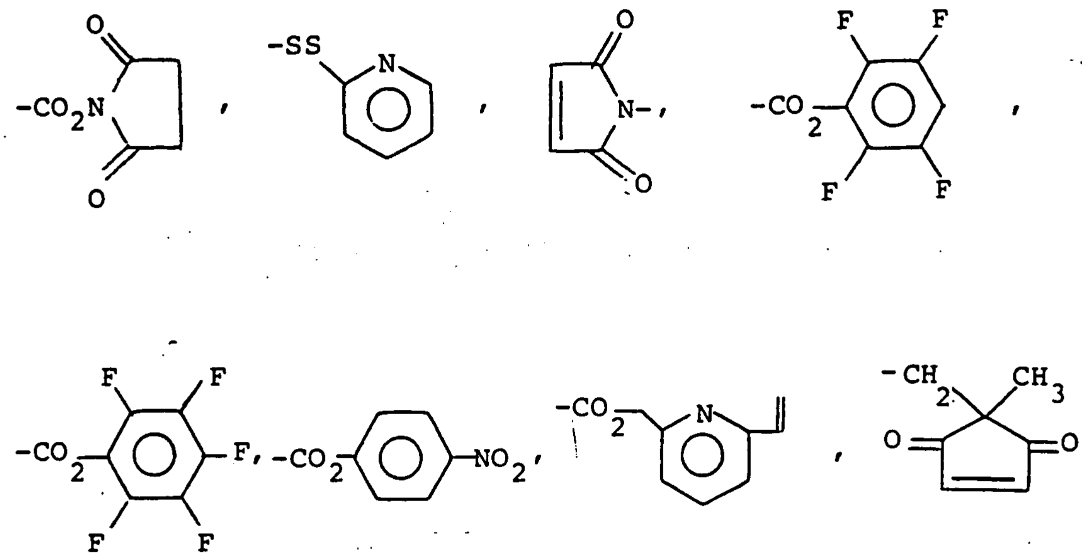
As can be seen from the structures disclosed above, the $\alpha_1$, $\alpha_2$, $\alpha_3$, $\alpha_4$, $\beta_1$, $\beta_2$, $\gamma_1$ and $\delta$ components of the LL-E33288 complex, as well as the BBM-1675, FR-900405, FR-900406, PD 114759, PD 115028, CL-1577A, CL-1577B, CL-1577D, CL-1577E and CL-1724 antibiotics each contain a methyltrithio group in their structure. The methyltrithio moiety of the above-named antibiotics is subject to displacement by a variety of thiol-containing organic molecules resulting in the formation of a new class of anticancer and antibacterial agents.

It has now been discovered that the displacement of the methyltrithio unit of the compounds listed in Table 1 and as depicted in Scheme I can be used to introduce a spacer (Sp), the judicious choice of which enable the introduction of targeting units into the compounds of the above-named patents and applications.

$$CH_3SSS\text{-}W \xrightarrow{Q\text{-}Sp\text{-}SH} Q\text{-}Sp\text{-}SS\text{-}W$$

Scheme I

wherein Sp is a straight or branched-chain divalent ($C_1$-$C_{18}$) radical, divalent aryl or heteroaryl radicals, divalent ($C_3$-$C_{18}$) cycloalkyl or heterocycloalkyl radicals, divalent aryl- or heteroaryl-alkyl ($C_1$-$C_{18}$) radicals, divalent cycloalkyl- or heterocycloalkyl-alkyl ($C_1$-$C_{18}$) radicals, or divalent ($C_2$-$C_{18}$) unsaturated alkyl radicals; Q is, or can be subsequently converted to, halogen, amino, alkylamino, carboxyl, carboxaldehyde, hydroxy, thiol, $\alpha$-haloacetyloxy, lower alkyldicarboxyl, $-CONHNH_2$, $-NHCONHNH_2$, $-NHCSNHNH_2$, $-ONH_2$, $-CON_3$,



and W is as shown in Table 1, above.

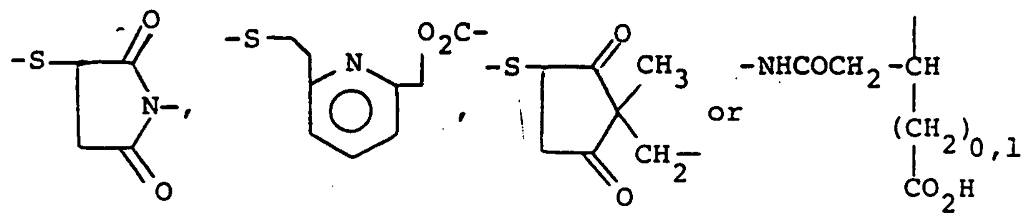
As long as the product from Scheme I contains at least one functional group which can be converted to, or is directly reactive with a targeting unit (Tu), targeted forms of the antitumor antibiotics of the above-named patents and applications can be generated, as shown in Scheme II below:

$$\text{Q-Sp-SS-W} \xrightarrow{\text{Tu-(Y)}_n} \underset{(\text{Y})_{n-m}}{\underset{|}{\text{Tu}}}\text{-(Z-Sp-SS-W)}_m$$

Scheme II

wherein Q, Sp, and W are as hereinbefore defined, Tu is a mono- or polyclonal antibody, its fragments, its chemically or genetically manipulated counterparts, growth factors, or steroids; Y is a side-chain amino, carboxy, or thiol group of a protein, an aldehyde derived from carbohydrate residues, or an amidoalkylthio group; n is an integer of from 1 to 100; Z

is formed from covalent reaction of the groups Q and Y directly or after subsequent reduction and Z is -CONH-, -CONHN=CH-, $-CONHNHCH_2-$, -NHCONHN=CH-, $-NHCONHNHCH_2-$, -NHCSNHN=CH-, $-NHCSNHNHCH_2-$, -ON=CH-, -NH-, $-NHCH_2-$, -N=CH-, $-CO_2-$, $-NHCH_2CO_2-$, -SS-,



and m is 0.1 to 15.

As an example, and with reference to Scheme II, above, the 3-mercaptopropionic acid derivative of E-33288$\gamma_1^I$ ($Q=CO_2H$, $Sp=-CH_2CH_2-$), when converted to its activated hydroxysuccinimide form ($Q=CO_2Su$, $Sp=-CH_2-CH_2-$) can be used to react with some of the $\epsilon$-amino groups of lysine residues (e.g., Tu=monoclonal antibody, $Y=-NH_2$ wherein n=50-100 from available lysine residues), at a pH between 7.0 and 9.5 in aqueous buffered solutions at temperatures between 4°C to 40°C to produce targeted forms of the antibiotics attached at random sites along the protein backbone (Tu=monoclonal antibody, Z=-NHCO-, $Sp=-CH_2CH_2-$, m=1-10). Only a fraction of the available lysine residues are substituted in this manner, since high loading is generally not considered compatible with preserving the antibody immunoreactivity. The same randomly-substituted immunoconjugates can also be prepared from the 3-mercaptopropionic acid derivative using other carboxyl group activating agents such as a variety of carbodiimides, or the corresponding acyl azide. Alternatively, a 3-mercaptopropionyl hydrazide derivative of E-33288$\gamma_1^I$

($Q=H_2NNHCO-$, $Sp=-CH_2CH_2-$), when reacted with a periodate-oxidized antibody (Tu=monoclonal antibody, Y=-CHO, n=1-15) as described in U.S. Patent No. 4,671,958 at a pH between 4 and 7, in a buffered aqueous solution at a temperature of between 4°C and 40°C, reacts only at the aldehyde functionality (derived from cleavage of vic-diols of carbohydrate residues situated on the Fc portion of the antibodies) to generate monoclonal antibody conjugates containing the drug substituted at specific sites along the backbone of the protein (Tu=monoclonal antibody, Z=-CH=NNHCO-, $Sp=-CH_2CH_2-$, m=0.5-10). In order to block unreacted aldehyde groups on the antibody and thus avoid crosslinking, as well as stabilize the hydrolytically labile Schiff's base linkages, it is preferable (though not essential) to react the latter conjugate first with a compound such as acetyl hydrazide or tyrosine hydrazide, then reduce with sodium cyanoborohydride or sodium borohydride to produce the stabilized constructs of this invention (Tu=monoclonal antibody, $Z=-CH_2NHNHCO-$, $Sp=-CH_2CH_2-$, m=0.5-10). Other aldehyde-reactive groups as part of the drug construct are within our invention to generate the products of Scheme II. Such functional groups are preferably, though not limited to, those which react with aldehydes under acidic aqueous conditions. The reactivity of protein lysines under basic conditions is sufficiently great such that their amines compete with the products of Scheme II for available aldehydes of the monoclonal antibody. Alternative aldehyde-reactive groups are, for example, the semicarbazide, the thiosemicarbazide, and the O-substituted hydroxylamine functionalities.

Assembly of targeted forms of the compounds listed in Table 1 is not restricted to the sequence outlined in Scheme II. The targeting unit (Tu) can be first modified to contain a thiol group, which is then reacted with the compounds of Table 1, in accordance with Scheme III below:

$$Tu(Y)_n \;+\; Q\text{-}Sp\text{-}S\text{-}P \longrightarrow \underset{(Y)_{n-m}}{Tu}\text{-}(Z\text{-}Sp\text{-}SH)_m$$

$$\Big\downarrow\; CH_3\text{-}SSS\text{-}W$$

$$\underset{(Y)_{n-m}}{Tu}\text{-}(Z\text{-}Sp\text{-}SS\text{-}W)_m$$

Scheme III

wherein Tu, Y, Q, Sp, W, n, and m are as hereinbefore defined, and P is hydrogen or 2-(pyridylthio), with the proviso that when Y is a thiol derived from a backbone amino acid residue of Tu, Z-Sp taken together is a covalent bond.

As an example, and with references to Scheme III, above, a monoclonal antibody can be reacted with 3-(2-dithio-pyridyl)propionic acid hydroxysuccinimide ester to modify the protein through lysine residues (Tu=monoclonal antibody, $Y=NH_2$, n=50-100, $Q=-CO_2Su$, $Sp=-CH_2-CH_2-$, P=2-pyridylthio). Following reduction with, for example, dithiothreitol, an intermediate is generated (Tu=monoclonal antibody, Z=-NHCO-, $Sp=-CH_2CH_2-$, m=1 to 15) which can be reacted with the compounds of Table 1 to generate the subject immunoconjugates. Similarly, 2-iminothiolane can be reacted with a monoclonal antibody to introduce thiol groups onto the surface of the protein directly, without requiring a reduction step (Tu=monoclonal antibody, Z=-NHCO-, $Sp=-(CH_2)_3-$, m=1 to 15), and this intermediate can be reacted with the compounds of Table 1 as before. Alternatively, sulfhydryl groups inherent within the structure of monoclonal antibodies in dimeric form as cystine residues can be used to participate in the reaction of Scheme III directly. Such sulfhydryls are traditionally exposed by a combination of enzymatic digestion and reduction of native monoclonal antibodies (Tu=Fab′ fragment, Z-Sp=bond, Y=SH), but the use of genetically-altered

constructs of monoclonal antibodies containing unpaired cystine residues is likewise contemplated.

A preferred embodiment of this invention is a protein-drug conjugate of the formula:

$$\underset{\displaystyle (Y)_{n-m}}{\underset{|}{Tu}}\text{-}(Z\text{-}Sp\text{-}SS\text{-}W)_m$$

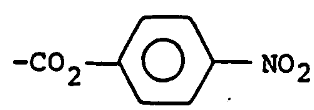
prepared from the antitumor antibiotic designated LL-E33288$\gamma_1^I$ ($CH_3SSS$-W) having

a) ultraviolet spectrum as shown in Figure I;

b) a proton magnetic resonance spectrum as shown in Figure II;

c) an infrared spectrum as shown in Figure III; and

displacing the dithiomethyl moiety with a compound of formula Q-Sp-SH, wherein Sp is straight or branched-chain divalent ($C_2$-$C_5$) radicals or divalent ($C_2$-$C_5$) arylalkyl or heteroarylalkyl radicals, and Q is carboxyl, lower alkyldicarboxyl anhydride, $-CO_2Su$, $-CONHNH_2$, or

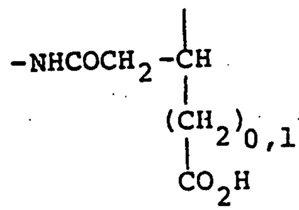


to produce an intermediate of general formula Q-Sp-SS-W, wherein Q, Sp, and W are as hereinbefore defined,

reacting Q-Sp-SS-W with a molecule of the formula Tu-$(Y)_n$ wherein Tu is a monoclonal antibody which exhibits preferential reactivity with a human tumor-associated antigen, Y is a side-chain amino group on the antibody, or an aldehyde generated by oxidation of the carbohydrate groups of the antibody, and n is an integer of from 1 to 100, to produce a compound of the formula:

$$\underset{\displaystyle (Y)_{n-m}}{\underset{|}{Tu}}\text{-}(Z\text{-}Sp\text{-}SS\text{-}W)_m$$

wherein Tu, Y, Sp, W, and n are as hereinbefore defined, and Z is formed from covalent reaction of the groups Q and Y directly or after subsequent reduction, and Z is -CONH-, CONHN=CH-, $-CONHNHCH_2-$, or



and m is 1 to 15.

A number of different monoclonal antibodies (MoAb's) are used to exemplify targeting of the methyltrithio anticancer compounds. MoAb's Lym 1 and Lym 2 recognize different antigens on mature B-lymphocytes and their product lymphomas. The production and characteristics of these MoAb's are described by A.L. Epstein, et. al., "Cancer Research" 47, 830 (1987). MoAb B72.3 targets primarily to carcinomas of the breast and colon, through reactivity with pancreatic, ovarian, and lung carcinomas has also been noted. The antibody has been described by T.L. Klug, et. al., "Int. J. Cancer" 38, 661 (1986). MoAb CT-M-01, which recognizes primarily breast tumors is described in EPO application 86 401482.4 filed July 3, 1986 and MAC-68 is produced by a sub-clone of the hybridoma which produces CT-M-01, and recognizes both breast and colon carcinomas. Intermediates of the subject compounds useful for, and conjugates with these antibodies, are described in the experimental section. It should not, however, be construed that this patent is limited to or restricted by the aforementioned antibodies. Instead, the methodology is sufficiently general that it can be applied to all antibodies regardless of their class or isotype, their enzymatically-derived fragments, their chemically manipulated and stabilized fragments, as well as their respective chimeric and humanized counterparts. Nor are the targeting units restricted only to monoclonal antibodies. Other proteins, as well as small molecules for which receptors exist on target

tissues, are within the purview of our discovery as targeting entities.

The methods of this invention used to produce monoclonal antibody conjugates from the compounds of Table 1 yield constructs which retain good immunoreactivity with target cell lines, as determined by the following in vitro assays:

Target Cells

All target cells were maintained in RPMI 1640 media supplemented with 5% Fetal Calf Serum (FCS), ITS (Collaborative Research, Cat# 40351), streptomycin (50 μg/ml), penicillin (50 units/ml), gentamycin sulfate (50 μg/ml) and glutamine (.03%). The cells were maintained in a humidified 5% $CO_2$ incubator at 37°C.

I. Immunoreactivity Assays

Procedure I - Elisa

Appropriate target cells were harvested, counted and suspended in Dulbecco's Phosphate Buffered Saline (DPBS) at an optimal concentration for monoclonal antibody (MoAb) being tested. 0.1 ml of cells was aliquoted in each well of a sterile tissue culture polystyrene 96-well plate. The plates were centrifuged for 5 minutes at 1,000 RPM's and the supernatant was flicked off. Plates were air-dried overnight and may be stored at 4°C for up to 3 months.

Non-specific binding sites were blocked by adding 200 μl of 1% gelatin in DPBS per well and incubating the plate for 1 hour at 37°C in a humid incubator. (All subsequent incubations are done under similar conditions). The plates were washed once with 250 μl of 0.05% TWEEN-20 in DPBS (washing solution) using the automated ELISA washing system from Dynatech (Ultrawash II). Samples to be tested were diluted to make a final concentration of 3 μg/ml MoAb equivalents in 0.1% gelatin-DPBS. Six additional threefold serial dilutions were prepared from each 3 μg/ml sample and 100 μl was added to appropriate wells in triplicate. The bottom row of wells only received 100 μl of 0.1% gelatin as background. Plates were incubated for 45 minut s and then washed three

times. Alkaline phosphatase conjugated affinity purified goat anti-mouse immunoglobulins (Cappel Cat# 8611-0231) was diluted 1:125 in 0.1% gelatin and 100 $\mu$l was added to each well. Plates were incubated for 45 minutes and then washed three times. 200 $\mu$l of p-nitrophenyl phosphate substrate solution (see below) was added to each well. After 45 minutes at room temperature the reaction was stopped by the addition of 50 $\mu$l of 3M NaOH. The absorbance of the contents of each well was read at 405 nm in the automated spectrophotometer from Dynatech (EIA Autoreader # EL-310).

Substrate Diethanolamine Buffer (10%)

97 ml diethanolamine

800 ml water

0.2 grams $NaN_3$

100 mg $MgCl_2 \cdot 6H_2O$

The reagents were dissolved by continuous stirring and 1M HCl was added until the pH was 9.8. The total volume was made up to 1 liter with water and filter sterilized with a 0.2 $\mu$ filter. The buffer was stored in the dark at 4$^{o}$C. Immediately before use, p-nitrophenyl phosphate (Sigma, Cat# 104-40) was dissolved in the 10% diethanolamine buffer (must be at room temperature) to give a final concentration of 1 mg/ml.

Calculation of O. D. Values

The percentage binding of each sample was calculated by the following equation:

$$\frac{A-B}{C-B} \times 100 = \%\ \text{Binding}$$

A = Average O.D. of test sample

B = Average O.D. of background

C = Average O.D. of 3 $\mu$g/ml unmanipulated MoAb control

The % binding was plotted on the non-log scale of a semi-log graph and the MoAb concentration was plotted on the log scale. The $BD_{50}$ (i.e. dose of antibody needed to give 50% binding) of each test sample was derived from the graph

and the amount of retention of immunoreactivity was calculated by the following equation:

$$\frac{BD_{50} \text{ of MoAb control}}{BD_{50} \text{ of test sample}} \times 100 = \text{\% Immunoreactivity retained}$$

Procedure 2 - Indirect RIA

Appropriate amounts of target cells in 0.2 ml of 10% FCS media were aliquoted into 4 ml polystyrene tubes. Samples to be tested were diluted to a concentration of 2 μg/ml MoAb equivalents in 10% FCS media. Five three-fold serial dilutions were prepared from each 2 μg/ml sample and 0.2 ml was added to each tube in duplicate. Background samples received only cells and media. Cells were incubated at 4°C for 1 hour, then washed 2 times (all RIA washes were done with a 3 ml volume) with 2% FCS media. 0.05 ml of sheep $F(ab')_2$ anti-mouse IgG [$^{125}$I] (DuPont, Cat# NEX 162-0142) containing approximately 500,000 CPM's was added to each tube; cells were incubated an additional hour at 4°C, washed once with 2% FCS and twice with PBS. 0.5 ml of PBS was added to each tube, cells were vortexed, transferred to clean tubes and counted for 1 minute in a Packard Gamma 500.

The % binding of each value was determined and graphed like the preceding ELISA equation, except CPM's were substituted for O.D. units and C = Average CPM's of 1 μg/ml unmanipulated MoAb control. The % immunoreactivity retained of each sample was calculated as previously discussed.

Procedure 3 - Direct RIA

Appropriate amounts of target cells in 1 ml of 10% FCS media were aliquoted into 4 ml polystyrene test tubes, centrifuged and supernatant was discarded. Samples to be tested were diluted to make a concentration of 200 μg/ml MoAb equivalents in 10% FCS media. Five additional five-fold serial dilutions were prepared from each 200 μg/ml sample and 0.05 ml was added to each tube in duplicate. 0.05 ml of $^{125}$I-MoAb was added to each tube (optimal amount is indivi-

dually determined for each MoAb and batch). Positive control samples contained cells, media and $^{125}$I-MoAb. Background samples contained non-specific cells, media and $^{125}$I-MoAb. Cells were incubated for 1 hour at 4$^{o}$C, washed once with 2% FCS media, twice with PBS, transferred and counted as previously mentioned.

The % $^{125}$I-MoAb binding inhibition of each sample was calculated by the following formula:

$$\frac{A-B}{C-B} \times 100 = \% \ ^{125}\text{I-MoAb Binding inhibition}$$

A = Average CPM's of sample

B = Average CPM's of background

C = Average CPM's of positive control

The plot and % immunoreactivity retained by each sample was calculated as previously discussed except the $BD_{50}$ is actually $BID_{50}$ (Dose of MoAb needed to give 50% inhibition of the binding of $^{125}$I-MoAb).

<u>Notes</u>:

1) Tubes were always vigorously vortexed immediately after the addition of every reagent in the RIA's.
2) An internal control sample equalling 50% of the unmanipulated MoAb control was included in each set of assays to confirm whether each procedure was quantitative in predicting the conjugates' retention of immunoreactivity.

The results from these assays are tabulated below in Table 2.

Table 2

Immunoreactivity of MoAb Conjugates

| Non-specific conjugates using the product of example 3 with: | Preparation | Immunoreactivity % of unmodified MoAb control |
|---|---|---|
| Lym 1 | #1 | 15 |
| B72.3 | #1 | 70 |
| | #2 | 10 |
| Hydrazide of 3-mercaptopropionic acid disulfide analog of $E33288\gamma_1^I$ (example 4) conjugated to: | | |
| Lym 1 | #1 | 100 |
| | #2 | 87 |
| | #3 | 64 |
| | #4 | 80 |
| | #5 | 100 |

Table 2 (continued)

| Hydrazide of 3-mercapto-propionic acid disulfide analog of E33288$\gamma_1^I$ (example 4 conjugated to): | Preparation | Immunoreactivity % of unmodified MoAb control |
|---|---|---|
| Lym 2 | #1 | 57 |
| | #2 | 85 |
| | #3 | 39 |
| | #4 | 70 |
| B72.3 | #1 | 100 |
| | #2 | 90 |
| CT-M-01 | #1 | 60 |
| MAC-68 | #1 | 40 |
| | #2 | 28 |
| **Hydrazide conjugates prepared using the product of example 5 with:** | | |
| Lym 1 | #1 | 100 |
| | #2 | 100 |

The monoclonal antibody conjugates of this invention are active as anticancer agents. The assay described below for assessing *in vitro* cytotoxicity shows the dramatic preference of the constructs for target cell lines as opposed to non-target cells, and provides a measure of utility of targeted forms of the compounds compared to their non-targeted counterparts.

Cytotoxicity Assays

In Vitro

Samples to be tested were diluted to a concentration of 0.2 or 0.02 μg/ml of LL-E33288$\gamma_1^I$ equivalents

(starting concentration is dependent on cell line to be tested). Three additional five-fold dilutions were prepared from each original sample dilution and 0.2 ml was added to sterile 15 ml polystyrene tubes. At least one similar conjugate consisting of LL-E33288$\gamma_1^I$ and an irrelevant MoAb was included in each assay to determine specificity of the relevant conjugate. $10^5$ appropriate target cells in 0.2 ml of 10% FCS media were aliquoted into the tubes and vortexed. In addition, an identical test was performed utilizing irrelevant cells as targets to further confirm specificity of relevant conjugate. MoAb controls received only equivalent amounts of MoAb and positive control samples received only 10% FCS media.

Cells were incubated at 37°C for 7 minutes then washed 4 times with 8 ml of 2% FCS media. 0.1 ml of 10% FCS was added to each tube, cells were vortexed and 0.2 ml was aliquoted to each well of a sterile 96-well polystyrene tissue culture plate.

Plates were incubated for 2 days in a humidified 37°C incubator with 5% $CO_2$. One half of the media was removed and replaced with fresh media containing 2 $\mu$Ci/ml $^3$H thymidine (DuPont, NEN, Cat# NET-027). Incubation was continued for 24 hours, cells were frozen, thawed and harvested by a PHD cell harvester (Cambridge Technology, Inc.). Each sample was counted for 1 minute in a Beckman LS 5800 scintillation counter on Channel 1.

The % growth inhibition was calculated as follows:

$$\frac{\text{Average CPM of test value}}{\text{Average CPM of media control}} \times 100 = \text{\% Growth}$$

$$100 - \text{\% Growth} = \text{\% Inhibition}$$

The % inhibition was plotted on the non-log scale of a semi-log graph and the LL-E33288$\gamma_1^I$ concentration was plotted on the log scale. The $IC_{50}$ (concentration of LL-E33288$\gamma_1^I$ needed to give 50% inhibition) of each test sample

was derived from the graph and the amount of retention of cytotoxicity was calculated by the following equation:

$$\frac{IC_{50} \text{ of LL-E33288}\gamma_1^{I}}{IC_{50} \text{ of test sample}} \times 100 = \text{\% Cytotoxicity Retained}$$

The results from the <u>in vitro</u> cytotoxicity assay are tabulated below in Table 3.

<u>Table 3</u>

<u>In Vitro Cytotoxicity of MoAb Conjugates</u>

| | <u>MoAb</u> | <u>Preparation</u> | <u>Cytotoxicity</u> %$E33288\gamma_1^{I}$ | % product of Example 1 |
|---|---|---|---|---|
| Non-specific conjugates prepared using product of Example 3 with: | | | | |
| | Lym 1 | #1 | .9 | 11.3 |
| | B72.3 | #1 | .001 | |
| | | #2 | 1.4 | 3.8 |
| | | | %$E33288\gamma_1^{I}$ | % product of Example 4 |
| Hydrazide conjugates prepared using product of Example 4 with: | | | | |
| | Lym 1 | #1 | | 80 |
| | | #2 | 56 | 191 |
| | | #3 | 40 | 60 |

Table 3 (continued)

| | MoAb | Preparation | Cytotoxicity %E33288$\gamma_1^I$ | Cytotoxicity % product of Example 4 |
|---|---|---|---|---|
| Hydrazide conjugates prepared using product of Example 4 with: | | | | |
| | Lym 1 | (#3 Against non-targeted cells) | 0 | 0 |
| | Lym 2 | #1 | | 29 |
| | | #2 | 2 | 100 |
| | | #3 | 2 | 55 |
| | B72.3 | #1 | 0 | 0 |
| | | #2 | 0 | 0 |
| | MAC-68 | #1 | | 90 |
| | CTM-01 | #1 | 111 | 830 |

| | | | %E33288$\gamma_1^I$ |
|---|---|---|---|
| Hydrazide conjugates prepared using product of Example 5 with: | | | |
| | Lym 1 | #1 | 300 |
| | | #2 | 100 |

The following assay system was used to measure the *in vivo* activity of the conjugates of this invention.

*In vivo* tests for antitumor activity on drug-monoclonal antibody conjugates were done using human tumor xenographs in athymic (nude) mice.

Burkitt lymphoma (Raji) and myeloma (HS Sultan) cells were harvested from culture flasks and inoculated subcutaneously ($\geq$ 80 x $10^6$ Raji cells or 40 x $10^6$ HS Sultan cells) into test mice. Solid tumors, ovarian carcinomas (CA73, Ovcar-3) and breast carcinoma (MX-1) were propagated in athymic mice, removed, cut into 2 $mm^3$ fragments and implanted subcutaneously into test mice (5-8 fragments per mouse).

Drugs, monoclonal antibodies and drug-monoclonal antibody conjugates were administered intraperitoneally once each 3 days for 3 or 5 total injections starting on day 2, 3, 4, 6, 7 or 8 days after tumor implantation. Tumor measurements (the length and width of the tumor) were made by means of a Fowler ultra CAL II electronic caliper each 7 days for 4 or 5 weeks post tumor implantation. Tumor mass in mg was estimated from the formula:

$$\frac{\text{Length(mm)} \times \text{Width(mm)}}{2}$$

Tumor growth inhibition was calculated for each test group on a percent of control [mean mg of treated (T) divided by mean mg of control (C) x 100]. A T/C value $\leq$ 42% in groups with $\geq$ 65% surviving animals is considered necessary to demonstrate activity.

The results from this assay appear in Table 4.

Table 4

In Vivo Antitumor Testing Results

| | Dosage(mcg) | | Tumor Size | S/T |
|---|---|---|---|---|
| | MoAb | Drug | (T/C)%control | |
| Hydrazide of 3-mercaptopropionic acid disulfide analog of E33288$\gamma_1^I$ conjugated to Lym 2 | 14.5 | 0.26 | 12 | 5/6 |
| Hydrazide alone | - | 0.26 | 34 | 4/6 |
| MoAb Lym 2 alone | 14.5 | - | 32 | 6/6 |
| Mixture, hydrazide + MoAb Lym 2 | 14.5 | 0.26 | 20 | 5/6 |

ip treatment against human melanoma cell line H.S. Sultan, 3 injections starting on day 5 after tumor implantation, measurements given made on day 35 post-implantation

Table 4 (continued)

| | Dosage(mcg) MoAb | Dosage(mcg) Drug | Tumor Size (T/C)%control | S/T |
|---|---|---|---|---|
| Hydrazide of 3-mercapto-propionic acid disulfide analog of E33288$\gamma_1^I$ conjugated to MAC-68 | 15.5 | 0.25 | 39 | 7/7 |
| Hydrazide alone | - | 0.25 | - | 0/6 |
| MoAb MAC-68 alone | 31 | - | 78 | 6/6 |
| Mixture, hydrazide + MoAb MAC-68 | 15.5 | 0.25 | - | 0/6 |
| Melphalan (as positive control) | - | 10 | 43 | 6/6 |

ip treatment against human ovarian cancer line CA73, three injections started 3 days after tumor implantation, measurements given made on day 35 post-implantation

Table 4 (continued)

| | Dosage(mcg) | | Tumor Size | S/T |
|---|---|---|---|---|
| | MoAb | Drug | (T/C)%control | |
| Hydrazide of 3-mercapto-propionic acid disulfide analog of E33288$\gamma_1^I$ con-jugated to CT-M-01 | 8.75 | 0.25 | 14 | 4/6 |
| Hydrazide alone | - | 0.25 | - | 0/6 |
| MoAb CT-M-01 alone | 8.75 | - | 75 | 5/6 |
| Mixture, hydrazide + MoAb CT-M-01 | 8.75 | 0.25 | - | 0/6 |
| Vincristine (positive control) | - | 1.0 | 0 | 4/4 |

ip treatment against human breast cancer cell line MX-1, three injections started on day 2 following tumor implantation, measurements given made on day 35 post-implantation

Table 4 (continued)

| | Dosage(mcg) MoAb | Drug | Tumor Size (T/C)%control | S/T |
|---|---|---|---|---|
| Hydrazide of 3-mercaptopropionic acid disulfide analog of E33288$\gamma_1^I$ conjugated to B72.3 | 6.2 | 0.125 | 62 | 6/6 |
| Hydrazide alone | - | 0.125 | 85 | 6/6 |
| MoAb B72.3 alone | 6.2 | - | 96 | 6/6 |
| Mixture, hydrazide + MoAb B72.3 | 6.2 | 0.125 | 105 | 5/6 |
| E33288$\gamma_1^I$ (3 treatments) | - | 0.005 | 141 | 5/6 |
| Cis platinum (positive control, 3 treatments) | - | 3.0 | 6 | 6/6 |

ip treatments against human ovarian cell line OVCAR-3, five injections starting on day 4 after tumor implantation (unless otherwise noted), measurements given made on day 35 post-implantation

Table 4 (continued)

| | Dosage(mcg) MoAb | Dosage(mcg) Drug | Tumor Size (T/C)%control | S/T |
|---|---|---|---|---|
| Hydrazide of 3-mercapto-propionic acid disulfide analog of E33288$\gamma_1^I$ conjugated to Lym 1 | 27 | 0.26 | 6 | 3/6 |
| Hydrazide alone | - | 0.26 | 72 | 6/6 |
| MoAb Lym 1 alone | 27 | - | 72 | 6/6 |
| Mixture, hydrazide + MoAb Lym 1 | 13 | 0.13 | 61 | 4/6 |

ip treatment against human Burkitt lymphoma cell line Raji TC, three injections started on day 7 after tumor implantation, measurements given made on day 28 post-implantation

Th invention will be further described in conjunction with the following non-limiting xamples.

## Example 1

## 3-Mercaptopropionic Acid Disulfide Analog of LL-E33288$\gamma_1^I$

To a solution of 90 mg of LL-E33288$\gamma_1^I$ in 90 ml of acetonitrile was added 10.6 mg of 3-mercaptopropionic acid in 1 ml of acetonitrile. The solution was vortexed and then stored at -20°C for 6 days. The solvent was removed *in vacuo* and the residue chromatographed over 10 ml of silica gel in methylene chloride. The column was developed with 50 ml of methylene chloride, 50 ml of 4% methanol in methylene chloride and finally 100 ml of 8% methanol in methylene chloride. Evaporation of this last fraction gave a residue which was taken up in ethyl acetate with the aid of a little acetone and added dropwise to an excess of hexane. The precipitate was collected and dried, giving 39 mg of the desired product (FABMS, M+H 1394). Retention time on $C_{18}$ reverse phase HPLC: 18 minutes with 50% acetonitrile/0.1 M aqueous ammonium chloride. (LL-E33288$\gamma_1^I$: 8.0 minutes, ester hydrolysis product: 1.5 minutes)

## Example 2

## Reaction of LL-E33288$\gamma_1^I$ with the *p*-nitrophenyl ester of 3-mercaptopropionic acid

### (A) Preparation of *p*-nitrophenyl ester of 3-mercaptopropionic acid

Commercial 3-mercaptopropionic acid in methylene chloride containing a catalytic amount of concentrated sulfuric acid was treated with isobutylene for 20 minutes. The solution was then extracted with 1N sodium bicarbonate solution after which the methylene chloride solution was dried using anhydrous magnesium sulfate. The solution was then evaporated to a colorless mobile liquid which NMR and mass spectral data indicated was the S-*t*-butylmercaptopropionic acid, *t*-butyl ester.

An aliquot of this ester was refluxed with 6N hydrochloric acid in dioxane for 2.5 hours. The solvent was evaporated, ethyl acetate was added and this solution was extract d with sodium carbonate. The sodium carbonate extract was treated with 6N hydrochloric acid until the pH of the suspension was 2.0. The suspension was then extracted with ethyl acetate, the extract dried over anhydrous magnesium sulfate and the solvent evaporated to a colorless liquid which $^{1}H$ NMR and mass spectral data indicated was S-t-butylmercaptopropionic acid.

This compound was converted to the p-nitrophenyl ester by treatment with equimolar amounts of p-nitrophenol and dicyclohexylcarbodiimide in tetrahydrofuran for 4 hours. The dicyclohexyl urea by-product was removed by filtration and the filtrate was evaporated to an oil which was purified by passage over neutral silica gel using the solvent system hexane:methylene chloride (50:50). The pure p-nitrophenyl ester derivative was a faintly yellow, mobile oil.

The free mercaptan was unmasked by the following procedure. The S-t-butylmercaptopropionic acid p-nitrophenyl ester was dissolved in trifluoroacetic acid and a slight molar excess (10%) of mercuric acetate was added. The mixture was stirred for 30 minutes, then the trifluoroacetic acid was evaporated and the residue taken up in dimethylformamide. This solution was treated with hydrogen sulfide gas for 15 minutes, then the black mercuric sulfide was filtered off and the filtrate evaporated under reduced pressure to eliminate up to 99% of the dimethylformamide. The resultant slightly brownish mobile liquid was purified over neutral silica gel using hexane:methylene chloride (50:50). The major component was shown by $^{1}H$ NMR to contain a small amount of the t-butyl mercapto derivative. Analytical HPLC over two Perkin-Elmer Pecosphere $C_{18}$ columns in tandem [4.6 x 33 mm and 4.6 x 83 mm] using a gradient system of 37.5/62.5 to 47.5/52.5 of acetonitrile and 0.1M ammonium acetate buffer at pH 6.5 (acetic acid) over a 12 minute span indicated that the product was 88% of the p-nitrophenyl ester of 3-mercaptopropionic

acid and 10% of the less polar S-*t*-butylmercaptopropionic acid *p*-nitrophenyl ester. There was also a small amount of free *p*-nitrophenol present.

(B) Reaction of *p*-nitrophenyl ester of 3-mercaptopropionic acid with LL-E33288$\gamma_1^I$

A 100 mg portion of LL-E33288$\gamma_1^I$ was dissolved in 50 ml of acetonitrile. To this was added a solution of 25.7 mg of *p*-nitrophenyl ester of 3-mercaptopropionic acid in 1 ml of acetonitrile. The reaction was left at -20$^{\circ}$C for 48 hours. HPLC indicated the reaction was complete. The solution was evaporated to dryness and the residue taken up in 4-5 ml of ethyl acetate using sonication to effect solution. The mixture was filtered and the filtrate dripped into 45 ml of stirred hexane. The resultant faintly yellow solid was collected and dried under reduced pressure, giving 93 mg of the *p*-nitrophenyl ester of propionic acid derivative of LL-E33288$\gamma_1^I$ as established by $^1$H NMR. By FABMS the [M+H] ion appeared at M/Z=1515.

## Example 3

## N-Hydroxysuccinimidyl 3-mercaptopropionate disulfide analog of LL-E33288$\gamma_1^I$

To a solution of 5 mg of the 3-mercaptopropionic acid disulfide analog of LL-E33288$\gamma_1^I$ from Example 1 in 0.5 ml of tetrahydrofuran was added 0.45 mg of N-hydroxysuccinimide in 0.1 ml of tetrahydrofuran and then 1.8 mg of dicyclohexylcarbodiimide in 0.2 ml of tetrahydrofuran. The reaction was allowed to stir at room temperature for 4 hours and was then quenched with a large excess of hexanes. The solid was isolated by filtration and dissolved in ethyl acetate. The resulting solution was washed three times with brine, dried with magnesium sulfate, and evaporated to 5 mg of the desired product as a tan powder which was used without further purification. Retention time on reverse phase $C_{18}$ HPLC: 15 minutes with 40% acetonitrile/0.1 M aqueous ammonium chloride (starting material: 6.0 minutes).

## Example 4

## 3-Mercaptopropionyl hydrazide disulfide analog of LL-E33288$\gamma_1^I$

To 5.4 ml (3 eq) of anhydrous hydrazine in 100 ml of refluxing tetrahydrofuran under argon was added dropwise 9.2 ml (83 mmol) of methyl 3-mercaptopropionate in 50 ml tetrahydrofuran over 2 hours. The solution was refluxed an additional two hours, evaporated, and then diluted and evaporated twice from 300 ml of toluene. The product was applied to a plug of silica gel with 5% ethyl acetate/chloroform and eluted from the plug with 20% methanol/chloroform. The resultant 3-mercaptopropionyl hydrazide was a faintly pink oil which solidified when cooled but melted at room temperature.

To 50 mg of LL-E33288$\gamma_1^I$ in 50 ml of acetonitrile at -15°C was added 6.6 mg of 3-mercaptopropionyl hydrazide in 1 ml tetrahydrofuran. One equivalent of triethylamine and/or one equivalent of acetic acid was added as catalyst. The reaction was allowed to stir at 0°C for one hour and the solvent was then evaporated. The residue was chromatographed on silica gel with a 10-15% methanol in chloroform gradient to yield 26 mg of the desired product. Retention time on reverse phase $C_{18}$ HPLC: 5.0 minutes in 41% acetonitrile/0.1 M aqueous ammonium chloride.

## Example 5

## N-[[(4-Methyl-coumarin-7-yl)amino]acetyl]cysteine hydrazide disulfide analog of LL-E33288$\gamma_1^I$

A mixture of 1.0 g (5.7 mmol) of 4-methyl-7-aminocoumarin, 3.0 ml of ethyl bromoacetate (5 eq), 90 mg (0.1 eq) of sodium iodide, and 30 ml dimethylformamide was heated under argon at 80°C for 5 hours. The mixture was cooled, diluted with ethyl ether, washed three times with 50% brine, dried with magnesium sulfate, and evaporated to dryness. The crude product was dissolved in chloroform containing 1% ethyl acetate and filtered through a plug of silica gel. Recrystallization from diethyl ether containing a trace of chloroform yielded pure ethyl N-[(4-methyl-coumarin-7-yl)amino]acetate.

To 1.96 g (7.5 mmol) of the above ester in 15 ml of methanol and 15 ml of tetrahydrofuran was added 10 ml of 1N aqueous sodium hydroxide. After 30 minutes, 4 ml of 10% aqueous hydrochloric acid was added. The organic solvents were evaporated and the resultant crystalline product was filtered and washed with cold ethanol and then ether. This material was dissolved in 20 ml of tetrahydrofuran and 4 ml of dimethylformamide. Dicyclohexylcarbonyldiimidazole (1.3 g, 2.2 eq) was added and the reaction allowed to stir for 15 minutes. Cysteine ethyl ester hydrochloride (1.6 g, 2.5 eq) and triethylamine (1.2 ml) were then added. After a further three hours, the reaction was diluted with ethyl ether containing 5% methylene chloride and washed once with 10% aqueous hydrochloric acid and twice with brine. After drying with magnesium sulfate and evaporating the solvents, the crude product was crystallized by dissolving in chloroform containing a minimal amount of ethanol and then adding an excess of ether. The crystals were filtered and dried to give pure N-[[(4-methyl-coumarin-7-yl)amino]acetyl]cysteine ethyl ester.

A mixture of 5 ml of chloroform, 20 ml of methanol, and 0.4 ml of hydrazine hydrate were heated to reflux under argon. To this was added 550 mg of N-[[(4-methyl-coumarin-7-yl)amino]acetyl]cysteine ethyl ester. After refluxing for 9 hours the mixture was cooled and the solid product was filtered and washed with chloroform and then ethyl ether. The crude product (which contained thiol and disulfide) was dissolved in dimethylformamide containing dithiothreitol and triethyl amine. After 30 minutes the product was precipitated with excess ethyl ether and collected by filtration. This material was purified further by recrystallization from degassed acetonitrile containing dithiothreitol and a trac of triethyl amine to give pure N-[[(4-methyl-coumarin-7-yl)-amino]acetyl]cysteine hydrazide.

To 12 mg of 70% pure LL-E33288$\gamma_1^{I}$ in 12 ml acetonitrile at $0^{o}C$ was added 4 mg of N-[[(4-methyl-coumarin-7-yl)amino]acetyl]cysteine hydrazide in 1.2 ml dimethylformamide. After stirring overnight another 2 mg of N-[[(4-

methyl-coumarin-7-yl)amino]acetyl]cysteine hydrazide in 0.6 ml dimethylformamide was added. The reaction was stirred for 3 days at $0^{o}C$ and filtered. The acetonitrile was evaporated and the resultant dimethylformamide solution was diluted with an excess of 1:1 hexanes/ether. The product was isolated by filtration and further purified by chromatography on silica gel with a 15-20% gradient of methanol in chloroform to yield 3 mg of the desired product. Retention time on reverse phase $C_{18}$ HPLC: 3.5 minutes using 45% acetonitrile/0.1 M aqueous ammonium chloride.

## Example 6

## 3-Mercaptopropionyl hydrazide disulfide analog of LL-E33288$\alpha_3^I$

To 10 mg of LL-E33288$\alpha_3^I$ in 9 ml of acetonitrile at $-15^{o}C$ was added 6.6 mg of 3-mercaptopropionyl hydrazide in 1 ml acetonitrile. One equivalent of triethylamine and/or one equivalent of acetic acid were added as a catalyst. The reaction was allowed to stir at $0^{o}C$ for one hour and the solvent was then evaporated. The residue was chromatographed on silica gel with a 10-15% methanol in chloroform gradient to give the desired product. Retention time on reverse phase $C_{18}$ HPLC: 3.5 minutes in the system 45% acetonitrile/0.1 M aqueous ammonium chloride.

## Example 7

## Non-specific conjugation to proteins

The hydroxysuccinimide ester described in Example 3 was covalently attached to antibodies under slightly alkaline conditions. The following is a general procedure used to make the antibody conjugates listed in Table 5. Antibody at a concentration of 3-5 mg/ml in phosphate buffer containing 0.1M sodium chloride, pH 7.5 was reacted with a 5-20-fold molar excess of the product from Example 3 with stirring, at room temperature for from 1-4 hours. The conjugated protein was desalted chromatographically and aggregated protein was separated from monomeric material by gel filtration HPLC. Monomeric fractions were pooled and concentrated.

Table 5

Non-specific conjugates prepared using the product of Example 3

| MoAb | Drug Loading M/M |
|---|---|
| Lym 1 | 5.2 |
| B72.3 | 6.0 |
| B72.3 | 2.9 |

## Example 8

### Site-specific conjugate preparation

The general method for attaching hydrazide derivatives of drugs to oxidized antibodies is described in T. J. McKearn, et al., in U.S. Patent No. 4,671,958. The procedure has been applied to preparing antibody conjugates from the products of Examples 4 and 5 with specific modifications as described below. The products from these reactions and their characteristics are summarized in Table 6.

(A) Antibody Oxidation Antibody at a concentration of 5 to 10 mg/ml was dialyzed overnight against a 200 fold volume of 50mM sodium acetate buffer, pH 5.5 containing 0.1M sodium chloride (Buffer A). After dialysis, the MoAb was oxidized with 15mM to 200mM periodic acid in 0.2M sodium acetate. The oxidation was allowed to proceed in the dark, with stirring, at 4°C for 45 minutes after which time the oxidized MoAb was desalted on a ≥5 bed volume Sephadex G-25 column. The degree of oxidation of the antibody was assessed by reaction with p-nitrophenylhydrazine and comparing absorbance of the protein at 280mm vs. p-nitrophenylhydrazine at 395mm.

(B) Drug Hydrazide Conjugation The oxidized MoAb was reacted with 25 to 200-fold molar excess of drug hydrazide. The hydrazides were dissolved into dimethylformamide and added to the aqueous solution of MoAb. To avoid precipitation of MoAb, the final volume of dimethylformamide added did not exceed 10% of the total reaction volume. Reaction was allowed to proceed for 3 hours at room temperature, with stirring. To prevent crosslinking of unreacted aldehydes and subsequent

aggregation, a blocking agent, acetyl hydrazide was added in 100-fold molar excess three hours after addition of the drug hydrazide. To stabilize the Schiff's base linkage between aldehyde and drug hydrazide (a hydrazone), the product generally was reduced to an alkyl hydrazine by the addition of 10mM sodium cyanoborohydride, allowing the reaction to proceed for one more hour (total conjugation time - 4 hours). The conjugate was chromatographically desalted and exhaustively dialyzed (minimum time 48 hours) into pH 6.5 phosphate buffer for storage and testing.

Conjugates were analyzed for the presence of aggregates by gel filtration HPLC and for free drug by reverse phase HPLC. Drug loading was determined spectroscopically using the extinction coefficients of both the antibody and the drug to estimate molar concentrations of drug in conjugates.

Table 6

Hydrazide conjugates prepared from the product of Example 4

| MoAb | Preparation | Drug Loading M/M |
|---|---|---|
| Lym 1 | #1 | 1.4 |
| | #2 | 2.4 |
| | #3 | 1.0 |
| | #4 | 6.7 |
| | #5 | 3.3 |
| Lym 2 | #1 | 2.9 |
| | #2 | 1.9 |
| | #3 | 2.0 |
| | #4 | 2.8 |
| B72.3 | #1 | 2.3 |
| | #2 | 1.3 |
| CTM-01 | | 3.1 |
| MAC-68 | | 1.7 |
| Hydrazide Conjugates prepared from the product of Example 5 | | |
| Lym 1 | #1 | 0.15 |
| | #2 | 0.76 |

# CLAIMS

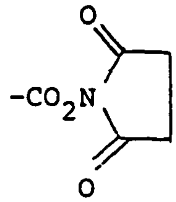
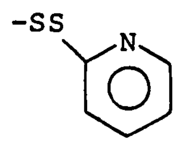
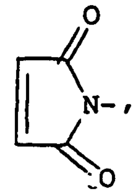
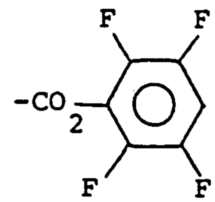
~~20,682-02~~ ~~We claim:~~

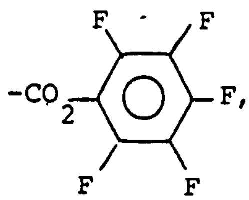
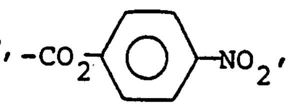
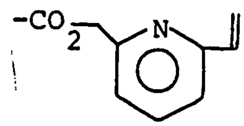
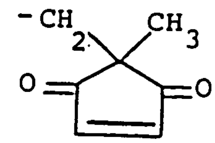
1. A carrier-drug conjugate of the formula

$$\begin{array}{l} \mathrm{Tu\text{-}(Z\text{-}Sp\text{-}SS\text{-}W)_m} \\ | \\ \mathrm{(Y)_{n-m}} \end{array}$$

prepared from a compound of formula $CH_3SSS$-W wherein $CH_3SSS$-W is an antitumor antibiotic designated as LL-E33288$\alpha_1^{Br}$, $\alpha_1^{I}$, $\alpha_2^{Br}$, $\alpha_2^{I}$, $\alpha_3^{Br}$, $\alpha_3^{I}$, $\alpha_4^{Br}$, $\beta_1^{Br}$, $\beta_1^{I}$, $\beta_2^{Br}$, $\beta_2^{I}$, $\gamma_1^{Br}$, $\gamma_1^{I}$, $\delta_1^{I}$, BBM-1675, FR-900405, FR-900406, PD 114759, PD 115028, CL-1577A, CL-1577B, CL-1577D, CL-1577E, or CL-1724 comprising:

reacting $CH_3SSS$-W with a compound of general formula Q-Sp-SH, wherein Sp is a straight or branched-chain divalent ($C_1$-$C_{18}$) radical, divalent aryl or heteroaryl radical, divalent ($C_3$-$C_{18}$) cycloalkyl or heterocycloalkyl radical, divalent aryl- or heteroaryl-alkyl ($C_1$-$C_{18}$) radicals, divalent cycloalkyl- or heterocycloalkyl-alkyl ($C_1$-$C_{18}$) radical or divalent ($C_2$-$C_{18}$) unsaturated alkyl radical, and Q is, or can be subsequently converted to, halogen, amino, alkylamino, carboxyl, carboxaldehyde, hydroxy, thiol, α-haloacetyloxy, lower alkyldicarboxyl, $-CONHNH_2$, $-NHCONHNH_2$, $-NHCSNHNH_2$, $-ONH_2$, $-CON_3$,

, ,  ,

  , 

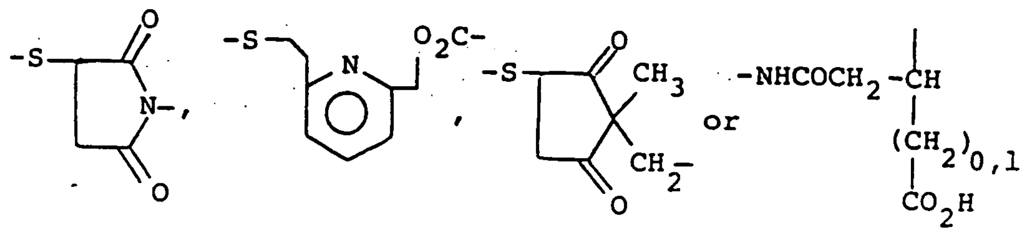
to produce an intermediate of formula Q-Sp-SS-W, wherein Q, Sp, and W are as hereinbefore defined,

reacting Q-Sp-SS-W with a molecule of the formula $Tu\text{-}(Y)_n$ wherein Tu is defined as a mono- or polyclonal antibody, its fragments, its chemically or genetically manipulated counterparts, growth factors, or steroids; Y is a side-chain amino, carboxy, or thiol group of a protein, an aldehyde derived from carbohydrate residues, or an amidoalkylthio group; and n is an integer of from 1 to 100, to produce a compound of the formula:

$$\begin{array}{l} Tu\text{-}(Z\text{-}Sp\text{-}SS\text{-}W)_m \\ | \\ (Y)_{n-m} \end{array}$$

wherein Tu, Y, Sp, W, and n are as hereinbefore defined, and Z is formed from covalent reaction of the groups Q and Y directly or after subsequent reduction, and Z is -CONH-, -CONHN=CH-, $\text{-CONHNHCH}_2\text{-}$, -NHCONHN=CH-, $\text{-NHCONHNHCH}_2\text{-}$,

-NHCSNHN=CH-, -NHCSNHNHCH$_2$-, -ON=CH-, -NH-, -NHCH$_2$-, -N=CH-, -CO$_2$-, -NHCH$_2$CO$_2$-, -SS-,



and m is 0.1 to 15.

2. A protein-drug conjugate of the formula

$$\begin{array}{l} Tu-(Z-Sp-SS-W)_m \\ | \\ (Y)_{n-m} \end{array}$$

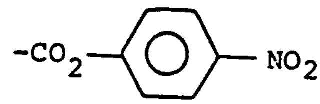
prepared from the antitumor antibiotic designated LL-E33288$\gamma_1{}^I$ ($CH_3SSS$-W) having

a) ultraviolet spectrum as shown in Figure I;

b) a proton magnetic resonance spectrum as shown in Figure II; and

c) an infrared spectrum as shown in Figure III; comprising:

displacing the dithiomethyl moiety with a compound of formula Q-Sp-SH, wherein Sp is straight or branched-chain divalent ($C_2$-$C_5$) radicals or divalent aryl- or heteroaryl-alkyl ($C_2$-$C_5$) radicals, and Q is, or can be subsequently converted to, carboxyl, lower alkyldicarboxylanhydride, -CONHNH$_2$, or

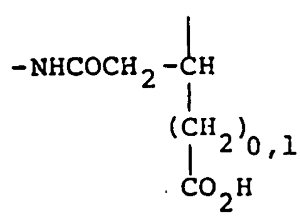


to produce an intermediate of general formula Q-Sp-SS-W, wherein Q, Sp, and W are as hereinbefore defined,

reacting Q-Sp-SS-W with a molecule of the formula $Tu-(Y)_n$ wherein Tu is a monoclonal antibody which exhibits preferential reactivity with a human tumor-associated antigen, Y is a side-chain amino group on the antibody, or an aldehyde generated by oxidation of the carbohydrate groups of the antibody, and n is an integer of from 1 to 100, to produce a compound of the formula:

$$\underset{(Y)_{n-m}}{\overset{|}{Tu}}-(Z-Sp-SS-W)_m$$

wherein Tu, Y, Sp, W, and n are as hereinbefore defined, and Z is formed from covalent reaction of the groups Q and Y directly or after subsequent reduction, and Z is -CONH-, -CONHN=CH-, $-CONHNHCH_2-$, or



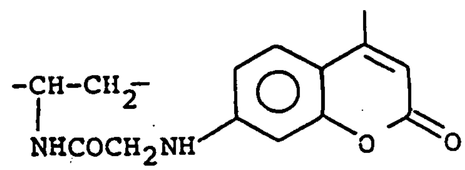
and m is 0.1 to 15.

3. A compound according to Claim 1 wherein $CH_3SSSW$ is $LL\text{-}E33288\gamma_1^{I}$, Q is the 4-nitrophenyl ester of a carboxyl group, Sp is $-CH_2CH_2-$, Tu is the monoclonal antibody CT-M-01, Y is $-NH_2$, Z is -CONH-, and m is 0.5 to 15.

4. A compound according to Claim 1 wherein $CH_3SSSW$ is LL-E33288$\gamma_1^{I}$, Q is the hydroxysuccinimide ester of a carboxyl group, Sp is $-CH_2CH_2-$, Tu is the monoclonal antibody MAC-68, Y is $-NH_2$, Z is -CONH-, and m is 0.5 to 15.

5. A compound according to Claim 1 wherein $CH_3SSSW$ is LL-E33288$\gamma_1^{I}$, Q is $-CONHNH_2$, Sp is $-CH_2CH_2-$, Tu is the monoclonal antibody Lym 1, Y is -CHO, Z is $-CONHNHCH_2-$, and m is 0.1 to 10.

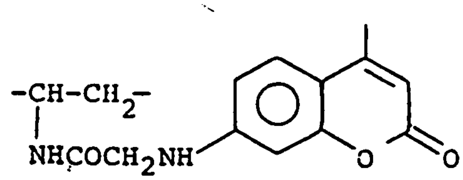
6. A compound according to Claim 1 wherein $CH_3SSSW$ is LL-E33288$\gamma_1^{I}$, Sp is




,

Tu is the monoclonal antibody B72.3, Y is -CHO, Z is $-CONHNHCH_2-$, and m is 0.1 to 10.

7. A compound according to Claim 1 wherein $CH_3SSSW$ is LL-E33288$\gamma_1^{I}$, Sp is




,

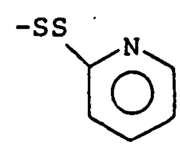
Tu is the monoclonal antibody Lym 2, Y is -CHO, Z is $-CONHNHCH_2-$, and m is 0.1 to 10.

8. A process for preparing the targeted derivatives

$$\begin{array}{l}Tu-(Z-Sp-SS-W)_m \\ | \\ (Y)_{n-m}\end{array}$$

of compounds of formula $CH_3SSS-W$, wherein $CH_3SSS-W$ is an antitumor antibiotic LL-E33288$\alpha_1^{Br}$, $\alpha_1^{I}$, $\alpha_2^{Br}$, $\alpha_2^{I}$, $\alpha_3^{Br}$, $\alpha_3^{I}$, $\alpha_4^{Br}$, $\beta_1^{Br}$, $\beta_1^{I}$, $\beta_2^{Br}$, $\beta_2^{I}$, $\gamma_1^{Br}$, $\gamma_1^{I}$, $\delta_1^{I}$, BBM-1675, FR-900405, FR-900406, PD 114759, PD 115028, CL-1577A, CL-1577B, CL-1577D, CL-1577E, or CL-1724, comprising

reacting $CH_3SSS-W$ with a compound of formula Q-Sp-SH, wherein Sp is a straight or branched-chain divalent $(C_1-C_{18})$ radical, divalent aryl or heteroaryl radical, divalent $(C_3-C_{18})$ cycloalkyl or heterocycloalkyl radical, divalent aryl- or heteroaryl-alkyl $(C_1-C_{18})$ radicals, divalent cycloalkyl- or heterocycloalkyl-alkyl $(C_1-C_{18})$ radical or divalent $(C_2-C_{18})$ unsaturated alkyl radical, and Q is halogen, amino, alkylamino, carboxyl, carboxaldehyde, hydroxy, lower alkyldicarboxyl anhydride, $-CONHNH_2$, $-NHCONHNH_2$, $-NHCSNHNH_2$, $-ONH_2$, or



in acetonitrile in the presence of one equivalent of triethylamine and/or one equivalent of acetic acid at $-10^{o}$ to $-30^{o}C$ for 1-48 hours,

isolating the intermediate of formula Q-Sp-SS-W, wherein Q, Sp, and W are as hereinbefore defined, then

reacting the compound of formula Q-Sp-SS-W, wherein Sp and W are as hereinbefore defined and Q is halogen, amino,

alkylamino, carboxyl, carboxaldehyde, hydroxy, or lower alkyl-dicarboxylic anhydride with a molecule of the formula $Tu\text{-}(Y)_n$ wherein Tu is a mono- or polyclonal antibody, its fragments, its chemically or genetically manipulated counterparts, growth factors, or steroids; Y is a side-chain amino or carboxy functionality; n is 1-100, in aqueous buffer at a pH of between 6.5 and 9, at $4^{\circ}$ to $40^{\circ}C$ either directly or in the presence of a water-soluble carbodiimide, to generate the compound

$$\begin{array}{l} Tu\text{-}(Z\text{-}Sp\text{-}SS\text{-}W)_m \\ | \\ (Y)_{n-m} \end{array}$$

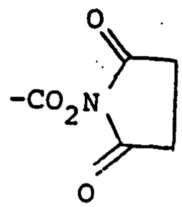
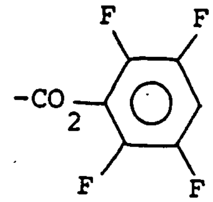
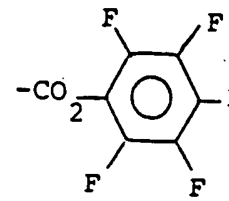
wherein Tu, Sp, W, n, and Y are as hereinbefore defined, m is 1-15 and Z is formed from covalent reaction of the groups Q and Y and is -CONH-, -NH-,

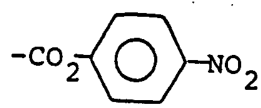
$$\begin{array}{l} \qquad\qquad\qquad\quad | \\ -NHCOCH_2\text{-}CH \\ \qquad\qquad\qquad\quad | \\ \qquad\qquad\qquad (CH_2)_{0,1} \\ \qquad\qquad\qquad\quad | \\ \qquad\qquad\qquad CO_2H \end{array}$$

-N=CH-, or $-CO_2-$

or

reacting the compound of formula Q-Sp-SS-W, wherein Sp and W are as hereinbefore defined and Q is a carboxylic acid, with N-hydroxysuccinimide, 2,3,5,6-tetrafluorophenol, pentafluorophenol, or 4-nitrophenol in the presence of a carboxyl activating agent such as a carbodiimide to generate a compound of formula Q-Sp-SS-W wherein Sp and W are as hereinbefore defined and Q is

 ,  , 

or 

with a molecule of formula Tu-$(Y)_n$,

where Tu and n are as hereinbefore defined, and Y is a side-chain amino group, in an aqueous buffered solution at a pH between 6.5 and 9, at a temperature of between 4° and 40°C, inclusive, to generate compounds of the formula:

$$\begin{array}{l} \text{Tu-(Z-Sp-SS-W)}_m \\ \;| \\ (Y)_{n-m} \end{array}$$

wherein Tu, Sp, Y, and n are as hereinbefore defined, m is 1-15, and Z is formed from covalent reaction between Q and Y and is defined as -CONH-

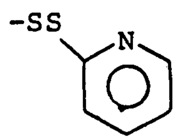
or

reacting a compound of formula Q-Sp-SS-W, wherein Sp and W are as hereinbefore defined and Q is $-CONHNH_2$ with nitrous acid in aqueous acetonitrile to generate a compound of formula Q-Sp-SS-W, wherein Sp and W are as hereinbefore defined and Q is $-CON_3$ with a compound of formula Tu-$(Y)_n$, wherein Tu, Y, and n are as hereinabove defined to produce a compound of the formula

$$\begin{array}{l} \mathrm{Tu\text{-}(Z\text{-}Sp\text{-}SS\text{-}W)_m} \\ \ \ | \\ \mathrm{(Y)_{n-m}} \end{array}$$

wherein Tu, Z, Sp, W, m, Y, and n are as hereinabove defined; or

reacting a compound of formula Q-Sp-SS-W wherein Sp and W are as hereinbefore defined and Q is hydroxy, with an alpha-haloacetic anhydride to produce a compound wherein Q is α-haloacetyloxy, and reacting the α-haloacetyloxy-Sp-SS-W or a compound of formula Q-Sp-SS-W, wherein Sp and W are as hereinbefore defined and Q is



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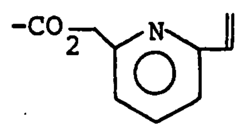
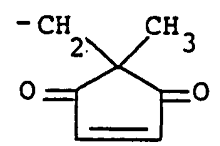


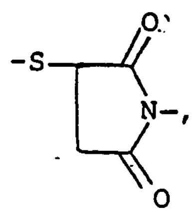
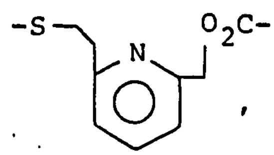
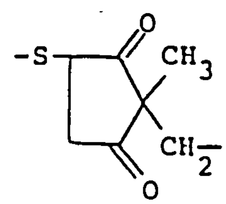
, or



with a molecule of the formula Tu-$(Y)_n$ wherein Tu is as hereinbefore defined, Y is a side-chain thiol of a protein, or an amidoalkylthio group introduced on an amine of Tu using reagents such as 3-(2-dithiopyridyl)propionic acid hydroxysuccinimide ester followed by reduction with an agent such as dithiothreitol, or an amidoalkylthio group introduced on an amine of Tu using 2-iminothiolane, and n is 1-10, under aqueous buffered conditions at a pH between 4.5 and 7, at a temperature between 4° and 40°C, inclusive, to produce a compound of formula:

$$\begin{array}{l} Tu-(Z-Sp-SS-W)_m \\ | \\ (Y)_{n-m} \end{array}$$

wherein Tu, Sp, W, and n are as hereinbefore defined, and Z is formed from covalent reaction of the groups Q and Y and Z is

-SS-,   , or 

and n is 0.1 to 10;

or

reacting a compound of the formula Q-Sp-SS-W wherein Sp and W are as hereinbefore defined and Q is $-NH_2$, $-CONHNH_2$, $-NHCONHNH_2$, $-NHCSNHNH_2$, or $-ONH_2$ with a molecule of formula $Tu-(Y)_n$ wherein Tu is as hereinbefore defined, Y is an aldehyde generated from carbohydrate residues on Tu by oxidation in the presence of an alkaline earth periodate, in an aqueous buffer at a pH between 4.0 and 6.5, at 4° to 40°C, inclusive, and n is 1 to 20 to generate a compound of formula:

$$\begin{array}{l} Tu\text{-}(Z\text{-}Sp\text{-}SS\text{-}W)_m \\ | \\ (Y)_{n-m} \end{array}$$

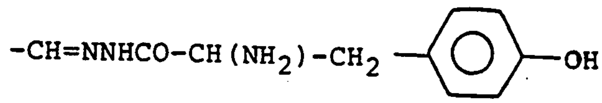
wherein Tu, Sp, W, Y, and n are as hereinbefore defined and Z is formed from the covalent reaction of Q and Y and is -ON=CH-, -N=CH-, -CONHN=CH-, -NHCONHN=CH-, or -NHCSNHN=CH-, and m is 0.1 to 15; or treating the compound immediately hereinabove of formula:

$$\begin{array}{l} Tu\text{-}(Z\text{-}Sp\text{-}SS\text{-}W)_m \\ | \\ (Y)_{n-m} \end{array}$$

wherein Tu, Z, Sp, W, Y, n, and m are as immediately hereinabove defined with acetylhydrazine or tyrosine hydrazine in an aqueous buffer at a pH between 4.0 and 6.5, at $4^{o}$ to $40^{o}C$, inclusive, to generate a compound of formula:

$$\begin{array}{l} Tu\text{-}(Z\text{-}Sp\text{-}SS\text{-}W)_m \\ | \\ (Y)_{n-m} \end{array}$$

wherein Tu, Z, Sp, W, n, and m are as immediately hereinabove defined and Y is $-CH{=}NNHCOCH_3$ or

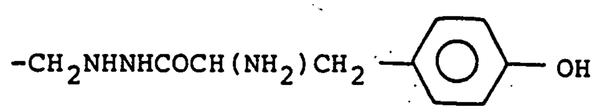


and

reacting this compound with sodium cyanoborohydride or sodium borohydride, in an aqueous buffer at a pH of 4.0 to 6.5, at a temperature of 4° to 40°C, inclusive, to generate a compound of formula:

$$\underset{\displaystyle (Y)_{n-m}}{\underset{|}{Tu}}-(Z-Sp-SS-W)_m$$

wherein Tu, Sp, W, m, and n are as hereinabove defined, Z is $-NH-CH_2-$, $-CONHNHCH_2-$, $-NHCONHNHCH_2-$, or $-NHCSNHNHCH_2-$, and Y is $-CH_2NHNHCOCH_3$ or

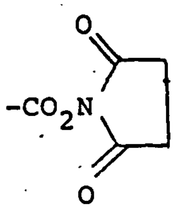
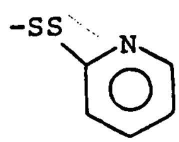
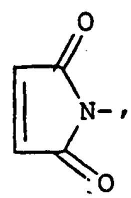
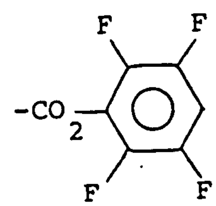


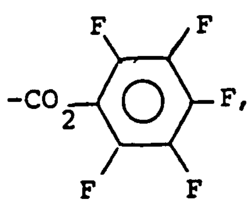
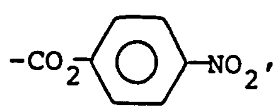
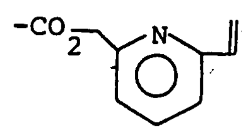
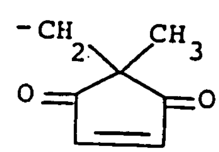
9. A method of inhibiting the growth of tumors in a mammal comprising administering to the mammal an oncolytic amount of a product

$$\underset{\displaystyle (Y)_{n-m}}{\underset{|}{Tu}}-(Z-Sp-SS-W)_m$$

prepared from a compound of general formula $CH_3SSS$-W wherein $CH_3SSS$-W is an antitumor antibiotic designated as LL-E33288$\alpha_1^{Br}$, $\alpha_1^{I}$, $\alpha_2^{Br}$, $\alpha_2^{I}$, $\alpha_3^{Br}$, $\alpha_3^{I}$, $\alpha_4^{Br}$, $\beta_1^{Br}$, $\beta_1^{I}$, $\beta_2^{Br}$, $\beta_2^{I}$, $\gamma_1^{Br}$, $\gamma_1^{I}$, $\delta_1^{I}$, BBM-1675, FR-900405, FR-900406, PD 114759, PD 115028, CL-1577A, CL-1577B, CL-1577D, CL-1577E, or CL-1724 comprising:

reacting $CH_3SSS$-W with a compound of general formula Q-Sp-SH, wherein Sp is a straight or branched-chain divalent $(C_1-C_{18})$ radical, divalent aryl or heteroaryl radical, divalent $(C_3-C_{18})$ cycloalkyl or heterocycloalkyl radical, divalent aryl- or heteroaryl-alkyl $(C_1-C_{18})$ radical divalent cycloalkyl- or heterocycloalkyl-alkyl $(C_1-C_{18})$ radical or divalent $(C_2-C_{18})$ unsaturated alkyl radical, and Q is, or can be subsequently converted to, halogen, amino, alkylamino, carboxyl, carboxaldehyde, hydroxy, thiol, α-haloacetyloxy, lower alkyldicarboxyl, $-CONHNH_2$, $-NHCONHNH_2$, $-NHCSNHNH_2$, $-ONH_2$, $-CON_3$,

, ,  ,

  , 

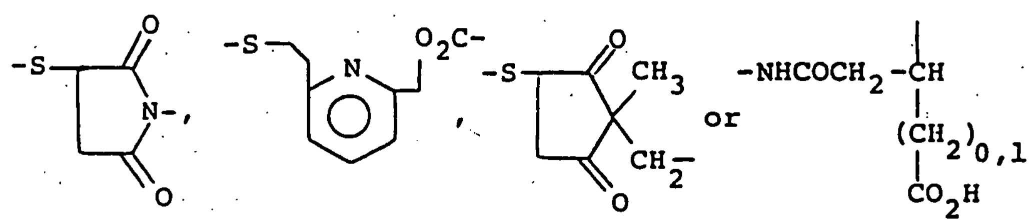
to produce an intermediate of formula Q-Sp-SS-W, wherein Q, Sp, and W are as hereinbefore defined,

reacting Q-Sp-SS-W with a molecule of the formula Tu-$(Y)_n$ wherein Tu is defined as a mono- or polyclonal

antibody, its fragments, its chemically or genetically manipulated counterparts, growth factors, or steroids; Y is a side-chain amino, carboxy, or thiol group of a protein, an aldehyde derived from carbohydrate residues, or an amidoalkylthio group; and n is an integer of from 1 to 100, to produce a compound of the formula:

$$\underset{(Y)_{n-m}}{\underset{|}{Tu}}\text{-}(Z\text{-}Sp\text{-}SS\text{-}W)_m$$

wherein Tu, Y, Sp, W, and n are as hereinbefore defined, and Z is formed from covalent reaction of the groups Q and Y directly or after subsequent reduction, and Z is -CONH-, -CONHN=CH-, $-CONHNHCH_2-$, -NHCONHN=CH-, $-NHCONHNHCH_2-$, -NHCSNHN=CH-, $-NHCSNHNHCH_2-$, -ON=CH-, -NH-, $-NHCH_2-$, -N=CH-, $-CO_2-$, $-NHCH_2CO_2-$, -SS-,



and m is 0.1 to 15.

10. A substance or composition for use in a method for _in vivo_ delivery of a compound at an antigenic site, said substance or composition comprising a protein-drug conjugate of the formula

$$\underset{(Y)_{n-m}}{\overset{|}{\mathrm{Tu}}}\text{-}(Z\text{-}Sp\text{-}SS\text{-}W)_m$$

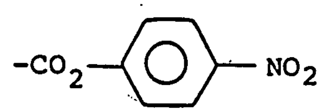
prepared from the antitumor antibiotic designated LL-E33288$\gamma_1^{I}$ ($CH_3SSS$-W) having

a) ultraviolet spectrum as shown in Figure I;

b) a proton magnetic resonance spectrum as shown in Figure II;
and

c) an infrared spectrum as shown in Figure III;
comprising:

displacing the dithiomethyl moiety with a compound of formula Q-Sp-SH, wherein Sp is straight or branched-chain divalent ($C_2$-$C_5$) radicals or divalent aryl- or heteroaryl-alkyl ($C_2$-$C_5$) radicals, and Q is, or can be subsequently converted to, carboxyl, lower alkyldicarboxylanhydride, $-CONHNH_2$, or



to produce an intermediate of general formula Q-Sp-SS-W, wherein Q, Sp, and W are as hereinbefore defined,

reacting Q-Sp-SS-W with a molecule of the formula Tu-$(Y)_n$ wherein Tu is a monoclonal antibody which exhibits preferential reactivity with a human tumor-associated antigen, Y is a side-chain amino group on the antibody, or an aldehyde generated by oxidation of the carbohydrate groups of the antibody, and n is an integer of from 1 to 100, to produce a compound of the formula:

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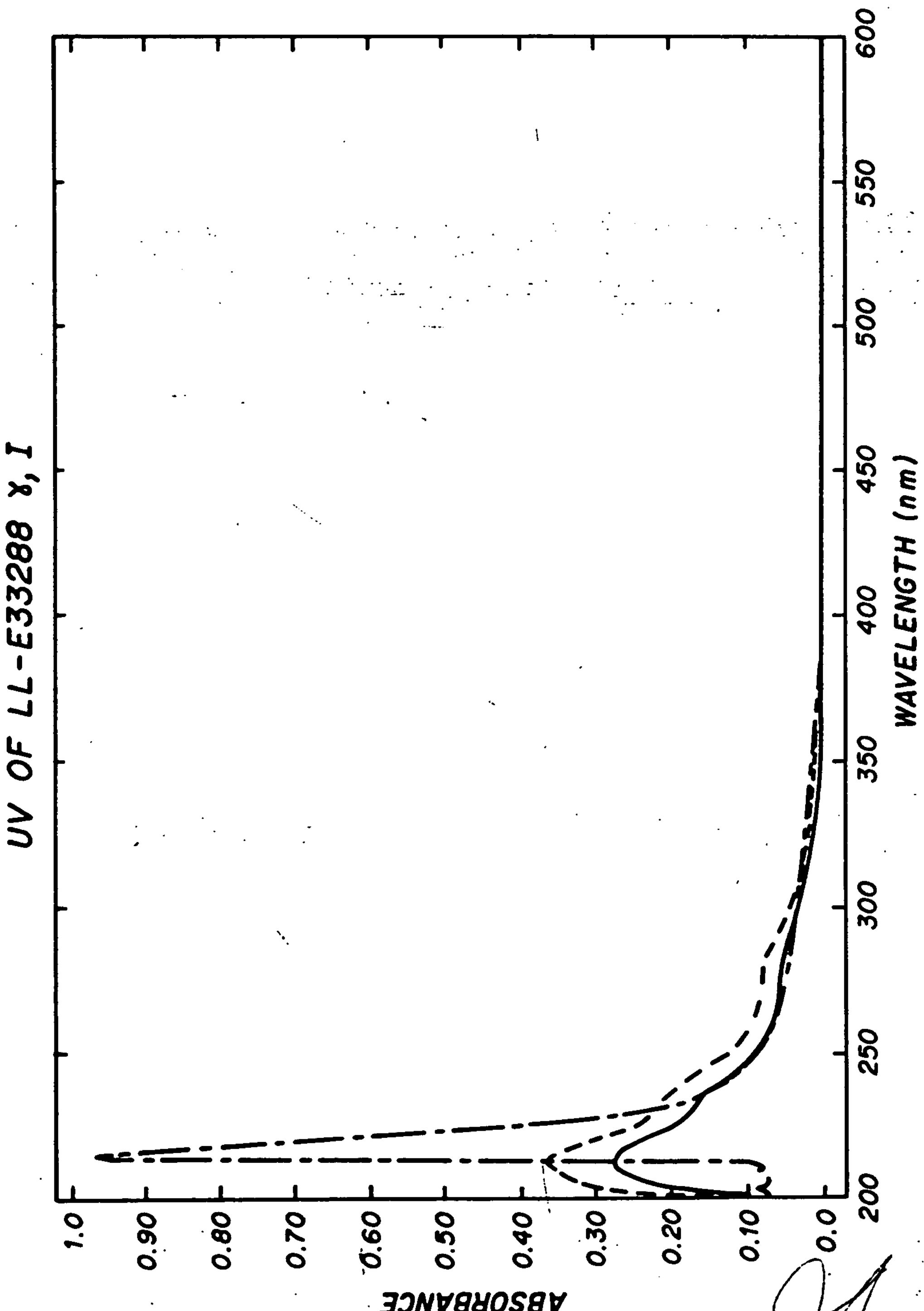


FIG. 1

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PMR OF LL-E33288, γ, I

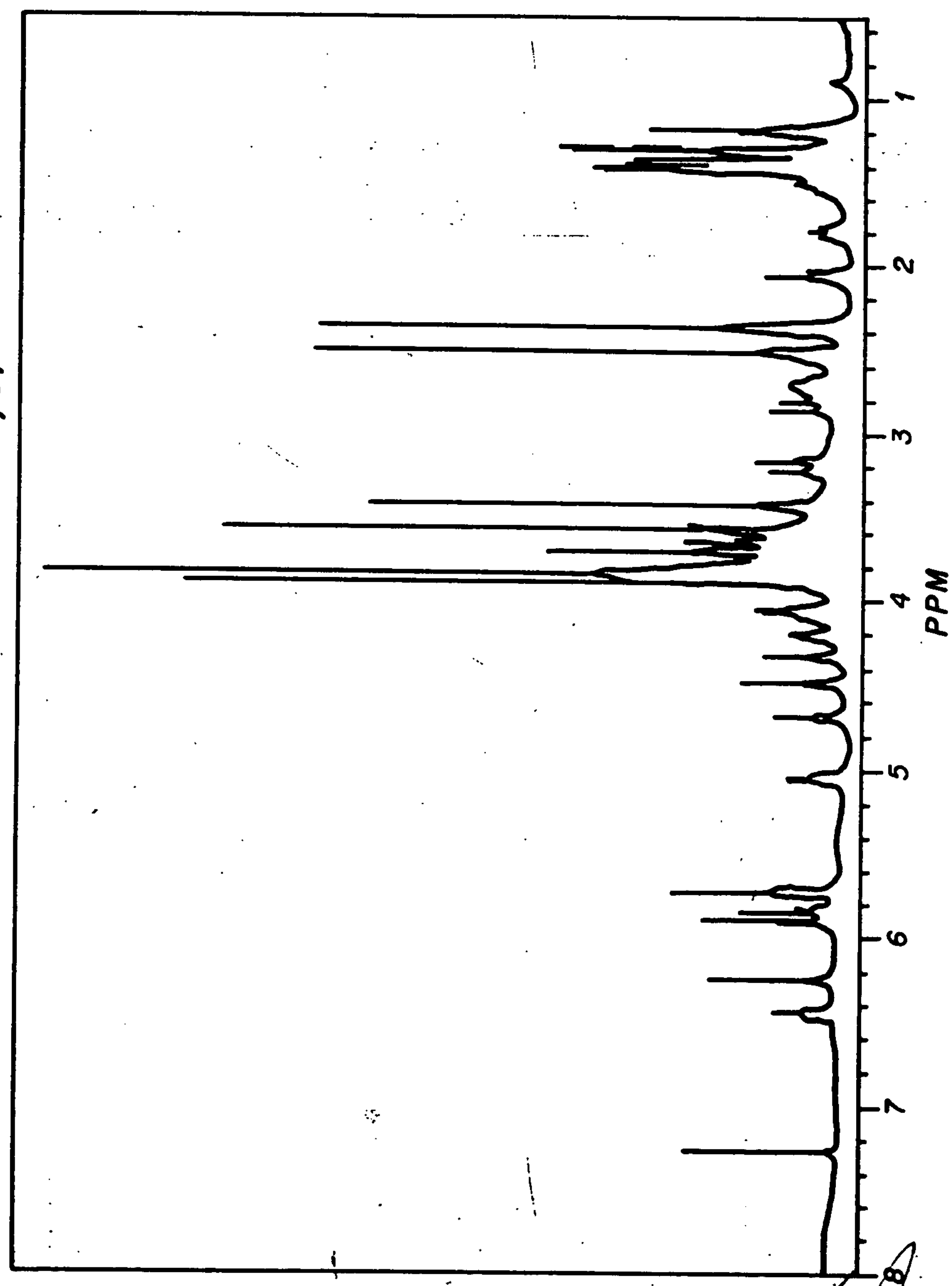


FIG. 2

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IR OF LL-E33288γ,I

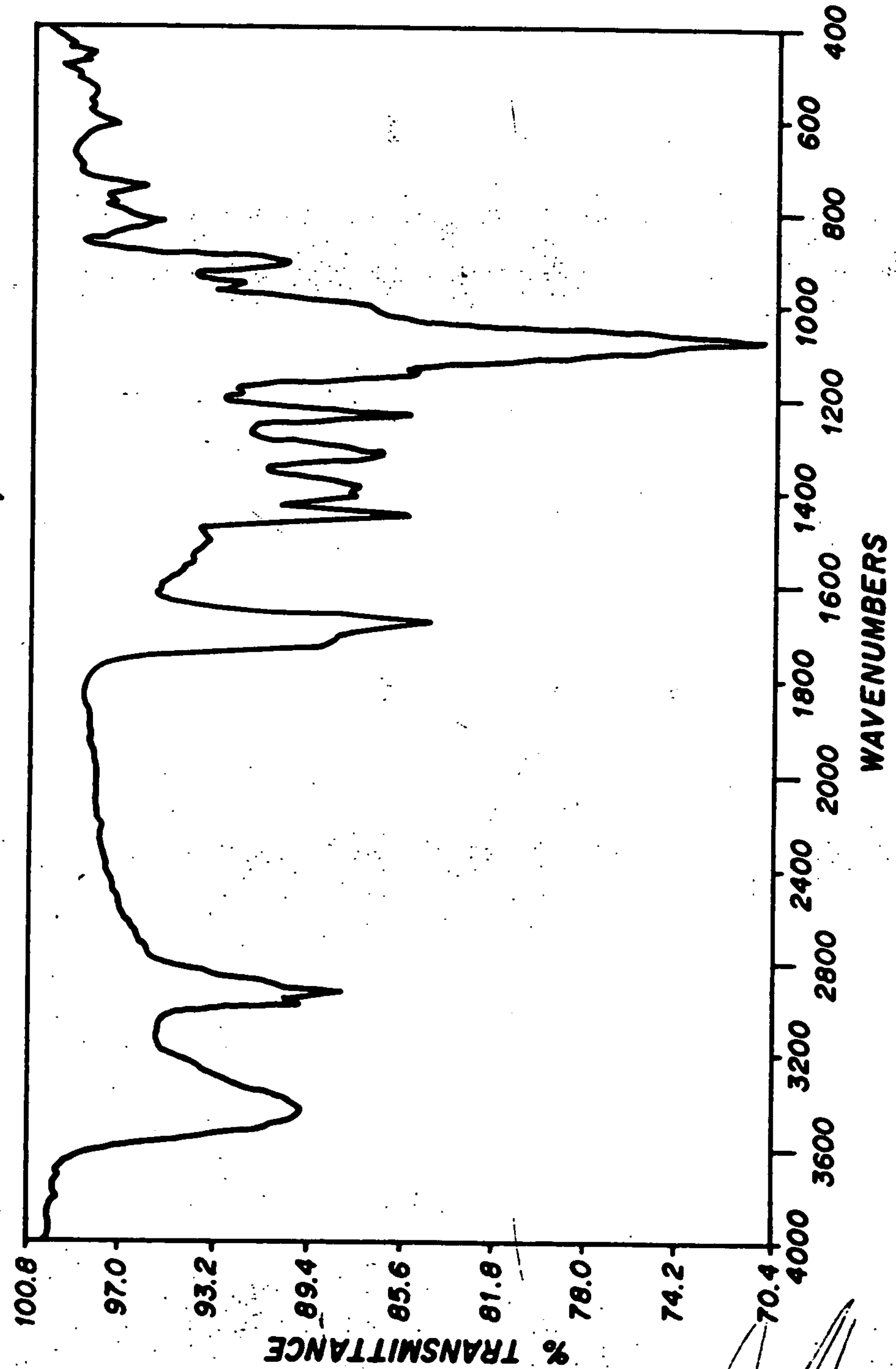


FIG. 3

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